Comparative Efficacy of Bactericidal Compounds in Buffer Solutions

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Verkürzte Fassung der

INAUGURALDISSERTATION

zur Erlangung der philosophischen Doktorwürde
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

HANS KASPAR HESS
aus Wald (Zürich)

Basel
Buchdruckerei E. Birkhäuser & Cie., AG.
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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag der Herren Professoren Dr. K. MEYER und Dr. J. TOMCSIK.

Basel, den 24. Juni 1958.

Prof. Dr. K. MEYER
Dekan

Herrn HANS KASPAR HESS wurde von der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel am 24. Juni 1958 erlaubt, diese verkürzte Fassung seiner am 24. Juni 1958 genehmigten Inauguraldissertation drucken zu lassen. Die vollinhaltliche Arbeit mit dem Titel:

„Vergleichende Wertbestimmung bakterizider Mittel
in gepufferten Lösungen"

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COMPARATIVE EFFICACY OF BACTERICIDAL COMPOUNDS IN BUFFER SOLUTIONS*

PART I

BY H. HESS† AND P. SPEISER

From the Institute of Pharmacy, University of Basle, Switzerland

Received June 2, 1959

A standardised membrane filter technique, adapted to the counting of organisms previously exposed to disinfectants, has been compared statistically with the pour-plate method. The membrane filters were found to be as suitable as pour-plates for counting viable organisms. The filter method is superior in disinfection experiments, since any quantity of test-mixture can be filtered and the danger of bacteriostasis is excluded. In disinfection experiments with high mortality levels, a great increse in variation occurred in replicate tests. Enriched media were not found to be superior to ordinary nutrient agar for the incubation of filter discs. The Gram-positive organisms tested showed a marked decrease in viable cells after storage at room temperature in slightly acid phosphate solutions for 24 hours, whereas in neutral and slightly alkaline solutions the viable count remained constant. The Gram-negative test organisms were not significantly affected by the phosphate buffer solutions.

THE object of this study was to compare the bactericidal activity of a number of disinfectants suitable for pharmaceutical solutions. A review of the literature has shown that there is little information available about the effect of the pH on the bactericidal activity of these compounds; for this reason, tests were made at four pH values from 4 to 8·5. The cell density chosen for the tests was such that the disinfectants were able, in concentrations used in pharmaceutical solutions, to sterilise the suspensions within periods varying between 30 minutes and 24 hours.

CHEMICAL

With three exceptions, the compounds were obtained commercially and complied with pharmacopoeial or analytical requirements or both: thus, no specifications are given for them. The following substances were tested.

Phenols. Phenol; *o*-, *m*-, and *p*-cresol; chlorocresol; *o*- and *p*-chlorophenol; *p*-nitrophenol; guaiacol (*o*-methoxyphenol); *p*-methoxyphenol; methyl, ethyl-, and propyl *p*-hydroxybenzoates; also *p*-ethoxyphenol prepared from *p*-phenetidin; the product, recrystallised from water, having a melting point of 65 to 66° (uncorr.) and a solubility of about 0·9 per cent w/v in water at 20°.

Aromatic alcohols. On different occasions commercial samples did not dissolve in water to give clear solutions even when saturation had not been reached. The impurities, presumably traces of hydrocarbons, could not

* This work forms part of a thesis submitted by one of us (H. H.) for the Degree of Doctor of Philosophy in the University of Basle.

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be removed by distillation. The process of purification was carried out as indicated below for 4-chloro-β-phenylethyl alcohol.

The alcohols used were, benzyl alcohol; β-phenylethyl alcohol; γ-phenylpropyl alcohol; phenoxetol (β-phenoxyethyl alcohol); Gecophen (4-chlorophenyl α-glycerol ether). Also included were 4-chlorobenzyl alcohol and 4-chloro-β-phenylethyl alcohol. The chlorobenzyl alcohol was prepared from 4-chlorobenzyl chloride; crystallisation from water gave the required alcohol in colourless needles of a m.p. of 66 to 68° (uncorr.) and b.p. of 116 to 120°/15 mm.; light absorption in ethanol: λ max 266 mμ (log ε 2·42); solubility: 0·35 per cent w/v of saline at 20° and 0·30 per cent at 3°. The chlorophenylethyl alcohol was prepared according to Harper and others[1]; purification of the product from traces of water-insoluble compounds was achieved by preparing the monoester of succinic acid and redistilling the saponified product, giving a colourless, virtually odourless liquid; b.p. 80–83°/0·07 mm.; light absorption in ethanol: λ max = 267 mμ (log ε 2·55); solubility: 0·48 per cent w/v in water at 20° and 0·46 per cent w/v in saline at 3°.

Organic mercury compounds. Phenylmercuric borate; its solubility is much better in slightly alkaline phosphate solutions; the solution of 0·022 per cent w/v in M/15 phosphate buffer of pH 4 and 5·5 is nearly saturated. Thiomersal.

Quaternary ammonium compounds. Domiphen bromide. Cetyl pyridinium chloride.

Experimental Methods

Buffer solutions. An isotonic phosphate buffer was used since Myers[2] pointed out that different buffer systems would exert different actions on *Escherichia coli.* It consisted of H_3PO_4, plus sodium phosphate with the appropriate amount of KCl added. Although the buffering capacity of this system is small at pH values of 4 and 8·5, it was sufficient for the purpose of the present tests. The phosphates and the phosphorus acid were dissolved under aseptic conditions in freshly distilled water from an all-glass still (Table I).

TABLE I

Preparation of isotonic phosphate buffer solutions

pH	M/15 Phosphorus acid + 0·645 per cent potassium chloride		M/15 Sodium dihydrogen phosphate $NaH_2PO_4 \cdot 2H_2O$ + 0·51 per cent potassium chloride		M/15 Disodium hydrogen phosphate Na_2HPO_4 + 0·38 per cent potassium chloride
4	8 ml.	+	992 ml.		
5·5			940 ml.	+	60 ml.
7			350 ml.	+	650 ml.
8·5*			15 ml.	+	985 ml.

* This solution has a pH of 8·55, after 2 days it falls to about 8·5.

Test solutions. The test solutions were prepared under aseptic conditions by dissolving the disinfectants in the buffer solutions, if necessary on a water bath. The solutions were filled, in amounts of 9 ml., into

aluminium-capped test-tubes, 15 × 160 mm., and inoculated with 1 ml. of bacterial suspension. Stock solutions in distilled water were prepared only from the quaternaries and from thiomersal. Phenols were dissolved in isotonic disodium hydrogen phosphate solution and the pH was adjusted as required. All pH readings were made with a glass : calomel electrode system.

Test organisms. Seven freshly isolated strains of *Escherichia coli* (type I, 44° C.-positive), three freshly isolated and one laboratory strain of *Pseudomonas pyocyanea*, and five freshly isolated strains of *Staphylococcus aureus*, were tested for their resistance to phenol. The most resistant strains were chosen, but results obtained with these were frequently checked with other strains. A culture of the *Staph. aureus* F.D.A. strain (No. 209) at our disposal was not sufficiently resistant and was used only in certain preliminary experiments; other strains also included one gravis and one atypical strain of *Corynebacterium diphtheriae*, one strain of *Serratia marcescens*, and one of *Streptococcus faecalis*.

Nutrient media. Cultures were maintained on agar slopes and sub-cultured every month; from these, new slopes were inoculated every week for test suspensions. The nutrient agar contained 0·5 per cent of peptone (Difco), 0·3 per cent of beef extract (Difco), 0·3 per cent of sodium chloride, 0·2 per cent of desiccated di-potassium hydrogen phosphate, and 2 per cent of agar; the pH was 7·4 to 7·5 before autoclaving at 120° for 20 minutes. The slopes for *C. diphtheriae* were enriched with 0·5 per cent of yeast extract (Difco) and an additional 0·5 per cent of peptone. With *Staph. aureus*, modifications of the nutrient agar did not induce a higher resistance to phenol. The agar was reduced to 1 per cent for the pour-plates and the media used in the disinfection experiments. In these, the nutrient agar was enriched with 0·5 to 1 per cent of yeast extract, an additional 0·5 per cent of peptone, 1 per cent of dextrose, and—when mercurials were tested—0·05 per cent of sodium thioglycollate.

Preparation of the test suspensions. The cultures were grown on slopes for 24 hours at 37° after which they were washed off with 5 ml. of water and the suspensions centrifuged at 3000 r.p.m. for 15 minutes. The centrifuged cells were resuspended in 5 ml. of phosphate buffer, pH 7, and shaken with small glass beads for 5 minutes to break up clumps. This washed suspension was diluted further with buffer so that the cell density in the final test-mixture was about 3×10^5. Plate counts made from each suspension revealed that the variation in the number of viable cells was not more than $\pm$ 50,000, and did not affect the results of the disinfection experiments.

The membrane filter technique employed. Cultivation of bacteria on membrane filters was first used for the bacteriological examination of water[3–5]; and subsequently for the testing of skin disinfectants[6–8], as well as for the evaluation of disinfection rates[9]. For the present study, we employed Co 5 membrane filters (Membranfiltergesellschaft, Göttingen) and the corresponding all-metal apparatus. Co 5 filters are nitrocellulose films of 120 μ thickness, the average "pore-diameter"[10] being 500 mμ and the filtering surface 12·5 sq. cm.

For the tests, the filters were "sterilised" in sterile hot water while the tubes with the disinfectant dilutions were placed in a water bath at either 20° ± 0·1° or 37° ± 0·1° half an hour before inoculation with the cell suspensions. After inoculation, the mouth of each tube was flamed and the tubes carefully shaken; this process was repeated once during the longer tests. Cotton-wool plugs are not suitable for the membrane filter method because exact counts cannot be made with accuracy owing to the presence of fibres on the filters. Before filtration, the filter unit was flamed, mounted while hot, and a quantity of sterile water was filtered to cool it. At intervals between 15 minutes and 24 hours (30 minutes, 2 hours, and 24 hours were most currently used), the whole test-mixture was filtered at 1 to 2 ml. per second, after which the filter was washed with 30 to 50 ml. of sterile water. The membrane was then removed and laid on the surface of a prepared nutrient agar plate. The plates were placed in the incubator within 5 minutes and incubated at 37° for 16 to 30 hours before reading. The concentration of agar in the medium was limited to 1 per cent to keep the surface as wet as possible The colonies so developing remain discrete and may be stained by incubating them on nutrient agar containing 0·05 per cent of potassium tellurite[7] for 2 to 4 hours; depending on the species, the colonies are stained brown or black. Counts were made on the dried filters by means of a magnifying lens.

Suitability of the Technique Employed

Influence of the Subculture Medium on the Number of Survivors

Several authors[11–13] have stressed the importance of enriched culture media for cells having survived lethal agents; this opinion, also advocated by Berry and Michaels[14], has not gone unchallenged[15]. We have tested several media with reference to their influence on the survivor count of *E. coli* and *Staph. aureus* by means of the membrane filter method, using phenols, quaternary ammonium germicides and organic mercury compounds and found no significant differences. These media contained various amounts of peptone, yeast extract, meat extract and glucose. The only difference observed was in the rate of growth. We may therefore infer that enriched media are growth-promoting but cannot "revive weakened cells". For this reason a medium containing nutrient agar, 1 per cent of peptone, 1 per cent of dextrose and 0·5 per cent of yeast extract was used in the tests.

Comparison of the Membrane Filter Technique and the Pour-plate Method

A statistical comparison was made with suspensions of *E. coli* and *Staph. aureus* in phosphate buffer pH 7·0 and suspensions of *Ps. pyocyanea* at pH 4·0, 5·5, 7·0 and 8·5. The cell density of the *E. coli* and the *Staph. aureus* suspensions was about 200/ml. and 140/ml. respectively, and the counts were made immediately. For *Ps. pyocyanea* the cell density was about 3×10^5/ml. and the counts were made after storage at 20° for 24 hours. During this time some growth of the organism had taken place, but this is taken into account in the figures recorded in Table II, which shows the comparative counts obtained by the two methods on suspensions

after suitable dilution. These growths probably account for the differences in counts recorded between the duplicate samples, and of the *Ps. pyocyanea* suspension.

TABLE II

COUNTS OBTAINED FROM PARALLEL EXPERIMENTS WITH MEMBRANE FILTERS AND POUR-PLATES

Experiment	Membrane filters (Co 5)				Pour-plates			
	Number of counts	Mean count (x)	Variance (s^2)	Standard deviation (s)	Number of counts	Mean count (x)	Variance (s^2)	Standard deviation (s)
534 (*Staph. aureus*) ..	18	142	90·9	9·5	19	155	221	15
535 (*E. coli*)	20	201	270·1	16	20	206	217·5	15
533 (*Ps. pyoc.*) immediate counts 8·5 a*	10	313	563·9	24	10	307	567·8	24
7 a	5	305	59·75	8	5	319	444·5	21
533 counts after 24 hours at 20° 8·5 a	5	206	461·75	21	6	170	583·6	24
8·5 b	5	70	88·5	9	7	61	13·1	4
7 a	5	129	163·7	13	5	111	193·5	14
7 b	5	95	119·5	11	5	79	51	7
5·5 a	5	102	212·25	15	5	79	150	12
5·5 b	5	92	49·25	7	5	72	92	10
4 a	6	100	149	12	5	113	128·5	11
4 b	4	79	74·3	9	5	88	143·5	12

* a and b are parallel tubes.

TABLE III

SIGNIFICANCE OF THE SUM OF THE PROBABILITIES RESULTING FROM THE t-TEST

Values of t obtained from experiments	Degrees of freedom	P
3·146	35	0·006
1·013	38	0·3
0·564	18	0·6
1·394	8	0·2
2·584	9	0·03
3·989	10	0·005
2·129	8	0·07
2·740	8	0·03
2·702	8	0·03
3·763	8	0·008
1·815	9	0·1
1·257	7	0·3

$\chi^2 = 2\Sigma\ (-\log_e P) = 70{\cdot}50$.
For 24 degrees of freedom (number of P multiplied by 2) $P < 0{\cdot}1$ per cent.

To obtain the counts, 1 ml. amounts were either distributed into petri dishes and plated in the usual way with nutrient agar or they were filtered on a membrane and the membrane was incubated on nutrient agar of the same composition. Each plate or filter was counted three times and individual counts were within $\pm$ 2 to 3 per cent of the mean. From these counts the variances of the two methods were calculated from the F values and they were found to be not significantly different. The t values were also calculated, but from these calculations it was not possible to decide whether the differences in the counts obtained by the two methods were due to chance. Subsequently, the data were subjected to further analyses by means of the combination of probabilities (Fisher[16]), and the findings from these are recorded in Table III. Using the Table of χ^2, we find a probability of less than 0·1 per cent for 24 degrees of

freedom. The difference between the means must therefore be regarded as highly significant. In the experiments with *Ps. pyocyanea*, all the significant differences appeared when the colony counts obtained with the filter method were higher, and it will likewise be seen that the cell density of the suspensions in question is low. From this statistical analysis it may be inferred that membrane filters are slightly superior when suspensions of low cell density have to be counted. This has been shown for *Ps. pyocyanea* with a probability of error of less than 0·1 per cent, but in our experience, it is also true for other test organisms, in spite of Wolochow's[17] opinions to the contrary. With higher counts, Co 5 filters should be rejected in favour of larger filters.

The Variation in Replicate Tests During the Course of Disinfection

Most authors making a quantitative recording of the number of survivors in disinfection experiments have found an excessive variation in replicate plates or roll-tubes[14]. In our experiments, a mortality level of 99·99 to 99·9999 per cent was currently achieved and so a larger set of replicates were filtered to examine in more detail the variations reported. These experiments were made under conditions as identical as possible, for example, using one test suspension for each series, the same batch of nutrient agar, membranes of the same serial number, and the disinfectant solutions were readjusted to pH 7·0. The initial counts were as usual $3 \cdot 10^5$ cells/ml., and 10 ml. amounts were filtered. Parallel with the larger series, two smaller ones were made also, representing the corresponding routine tests made in triplicate: the findings are summarised in Figures 1–4. Each of the Figures shows a strong divergence from a Poisson series, and this is the type of variation which has been encountered throughout this present study. It has compelled us to represent the results by means of a graphical method in which "mean counts"—represented by dotted lines—were computed from the logarithms of the single counts.

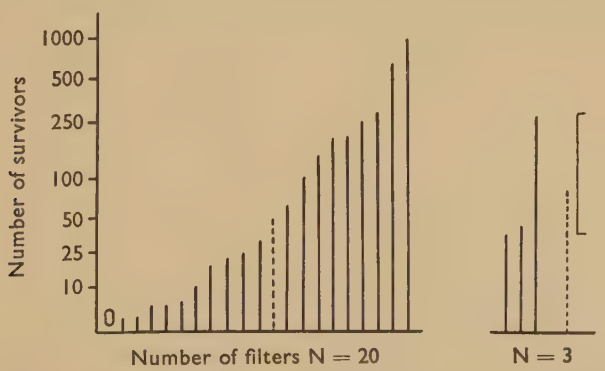

FIG. 1. Membrane filter counts of suspensions of *E. coli* in parallel tubes exposed for 2 hours at 20° to 0·3 per cent of *p*-cresol in isotonic phosphate buffer with a pH of 7·0. Left: Results of a larger series. Right: Results of a routine test. Dotted lines = "means", calculated from the logarithmic values. Each column represents the survivors of 10 ml. of test-mixture with an initial count of $3 \cdot 10^5$ cells/ml., 0 = sterile.

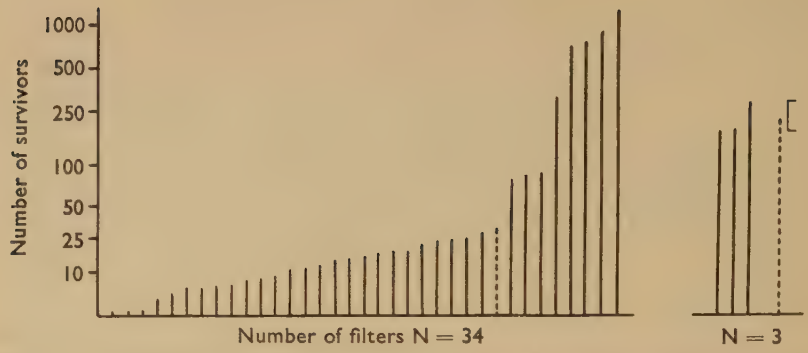

FIG. 2. Membrane filter counts of suspensions of *Staph. aureus* exposed for 24 hours at 20° to 0·2 per cent of 4-chloro-β-phenylethyl alcohol in isotonic phosphate buffer with a pH of 7·0. Left: a larger set of parallel filtrations was made. Right: Records the results of a corresponding routine test. Cell density and representation as in Fig. 1.

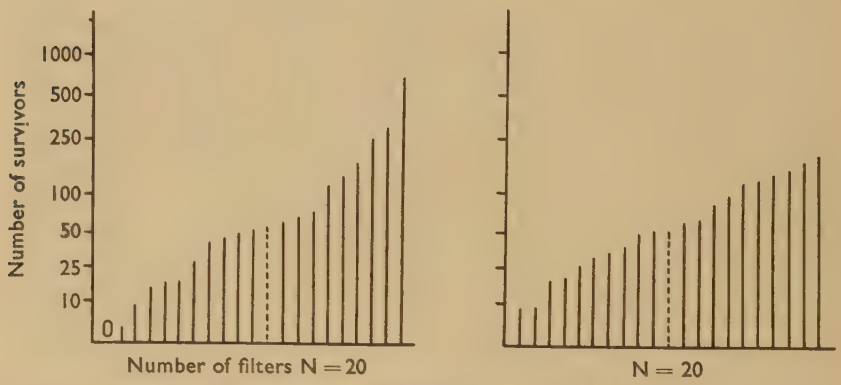

FIG. 3. Membrane filter counts of suspensions of *E. coli* (Left) and *Staph. aureus* (Right) exposed, for 30 minutes at 20°, to solutions of domiphen bromide 1 : 50,000 in isotonic phosphate buffer with a pH of 7·0. Cell density and representation as in Fig. 1.

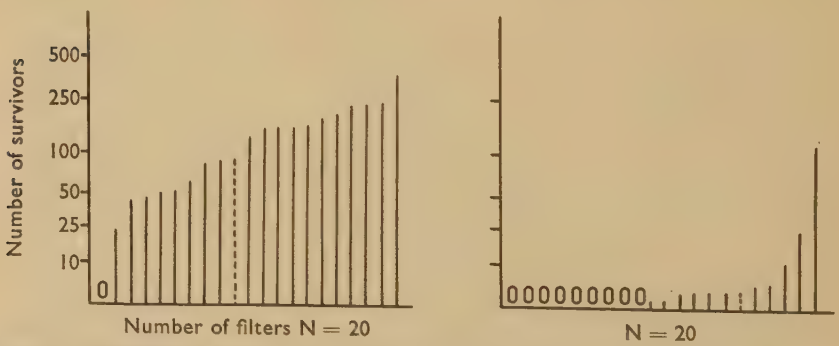

FIG. 4. Membrane filter counts of suspensions of *E. coli* (Left) and *Staph. aureus* (Right) exposed, for 30 minutes at 20°, to cetyl pyridinium chloride 1 : 100,000 in isotonic phosphate buffer with a pH of 7·0. Cell density and representation as in Fig. 1.

Viability of the Test Organisms in the Buffer Solutions

Suspension of the different organisms used were tested for their viability at different pH values at 20° for up to 24 hours; the number of viable cells was determined by plating, and sometimes parallel counts were made by means of membrane filters. Only the essential data are given here; for detailed results, see reference 18.

At a pH of 4, the mortality of *Ps. pyocyanea* was about 70 per cent and that of *E. coli* even less. Moderate growth occurred at the other pH values, with exception of *E. coli* at pH 8·5 where the viable count remained constant. *Staph. aureus* and *C. diphtheriae* were very sensitive to an acid pH; at pH 4, the suspensions were almost non-viable after 24 hours. The freshly isolated strains of *Staph. aureus*, used in the tests because of their higher resistance to phenol, were more easily killed by an acid pH than the F.D.A. strain at our disposal. At pH 7 and 8·5, the number of viable Gram-positive cells remained approximately constant.

Discussion

Statistical analysis has shown that the membrane filter technique adopted here is reliable when used to evaluate comparatively small numbers of viable cells in suspensions. The use of filters in disinfection experiments therefore has several obvious advantages: any quantity of test-mixture may be filtered, the filters may be washed to eliminate the danger of bacteriostat carry-over, except for mercurials, and colony growth occurs under uniform aerobic conditions.

The sensitivity of the membrane filter method is reflected in the results obtained with the three cresols. These are said to have equal bactericidal activity when measured with the phenol coefficient[19]. We have found, however, that *p*-cresol was most effective, closely followed by *o*-cresol, *m*-cresol being much less effective. The difference obtained can be ascribed only partly to the longer period of exposure, namely 30 minutes.

The disadvantages of end point methods in assessing disinfectants have been summarised by Berry[20], Berry and Michaels[21], Withell[22,23], and Wilson and Miles[24]. Nevertheless, in recent papers[25,26], end point methods have still been advocated because quantitative tests are greatly hindered by agglutination of the test suspension, and sterility has always been the desirable state. As for the counting methods, the mortality levels used therein have varied greatly. Jordan and Jacobs[27] preferred a very high mortality level of 99·999999 per cent ("virtual sterilisation time"), starting from an initial count of $3{\cdot}3 \times 10^8$. Other workers have suggested 99·9 or 99 per cent killing levels. Withell[22] introduced an LT50, the advantage being that bacteriostasis is avoided to a large extent in the subculture, and also that excessive variation in replicate tests is said to be reduced thereby. Jordan and Jacobs[27,28] found most discrepancies in their results when the mortality exceeded 95 per cent. Berry and Michaels[14] also observed some excessive variation in their disinfection experiments, even when using intermediate mortality levels, whereas for cells not having been in contact with disinfectants the distribution of

replicate counts followed a Poisson series. They tried to explain the difference by assuming that damaged organisms had more exacting growth requirements, and then went on to quote several authors in support of their thesis. As another possible explanation, Jordan and Jacobs have suggested that a certain percentage of the cells already damaged might be killed by the temperature of melted agar during the preparation of the plates or roll-tubes. Every heterogeneity of this type in colony formation would increase the distribution error already present and thus cause a rise in variation. These authors therefore suggested that a more adequate technique be adopted to handle the larger variation.

The membrane filter technique appears to be the answer to this problem. It yielded satisfactory results when untreated cells were counted; the danger of heat bacteriostasis is excluded since no heat is used, and, finally, growth takes place under equal conditions for all cells. Moreover, by filtering the whole test-mixture, the distribution error can be greatly reduced.

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COMPARATIVE EFFICACY OF BACTERICIDAL COMPOUNDS IN BUFFER SOLUTIONS

Part II

By H. Hess* and P. Speiser

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A number of phenols, aromatic alcohols, organic mercury compounds and quaternary ammonium compounds have been examined, by the membrane filter method, to assess their antibacterial action at four different pH values against various Gram-positive and Gram-negative organisms. The killing rate of the compounds tested varied with the pH. The phenols, in the undissociated state, and the aromatic alcohols were most effective against Gram-negative cells at a slightly alkaline pH, and least effective at a pH of 5·5 to 7, whereas against Gram-positive cells, the compounds were least bactericidal at a pH of 7 to 8·5. Upon Gram-positive as well as Gram-negative cells, phenylmercuric borate was more potent in alkaline solution, thiomersal, in acid solution. This difference is connected with the opposite charges of the active ions. A reduction in bactericidal activity was noted in acid solutions of domiphen bromide and cetyl pyridinium chloride although the activity of the latter was less influenced by pH. The importance of the partition ratio between water and a lipid phase for the bactericidal efficacy of phenols and aromatic alcohols is stressed, and a new approach to the testing of these compounds is proposed. 4-Chloro-β-phenylethyl alcohol yielded promising results as a new bactericidal agent. *Pseudomonas pyocyanea* was more rapidly killed by the compounds tested than *Escherichia coli*.

In Part 1 of this work[1], a technique for the counting of bacteria by means of membrane filters was described and analysed statistically. This technique has been used in a large number of experiments, the results of which are discussed in the following pages.

Experimental Methods

The details of the technique employed as well as the compounds and organisms tested have been described in Part I[1]. The cell density was 3×10^5/ml.; 10 ml. of the test-mixture was filtered through Co 5 membrane filters which were incubated on enriched nutrient agar with 0·05 per cent of thioglycollate for mercurials; three filter pads were used for each pH. Figures based on a logarithmic scale are used to present the counts, each column representing the "mean" of the three replicates, and the highest as well as the lowest count is indicated.

Results and Discussion†

Bactericidal Action of Phenols and Aromatic Alcohols

Influence of the pH. Figures 1 and 2 give the results obtained with *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas pyocyanea*, *Streptococcus faecalis*, *Serratia marcescens* and *Corynebacterium diphtheriae*

* Present address: c/o CIBA Limited, Basle.

† Only a small number of the counts obtained can be reproduced here. Values of n (concentration exponents) and Q_{10} (temperature coefficients), calculated on the basis of mortalities exceeding 99·9 per cent, may be summarised as follows: Phenols and aromatic alcohols: $n = 6$, $Q_{10} = 4{\cdot}3$ (calculated on the basis of the difference between 20° and 37°); mercurials: $n = 2$; quaternaries: $n < 2$.

tested against phenol and β-phenylethyl alcohol. For the Gram-negative species, the minimal action (that with most survivors) was found to be on the acid side, at about pH 5·5 for *E. coli*, and at pH 5·5 to 7 for *Ps. pyocyanea* and *Serr. marcescens*. For the Gram-positive cells, on the other hand, minimal action was at the slightly alkaline pH values of 7 to 8·5 whereas between pH 7 and 5·5, a large increase in bactericidal action was recorded. Similar results were obtained with the other phenols and aromatic alcohols tested; dissociating phenols, however, were found to behave differently.

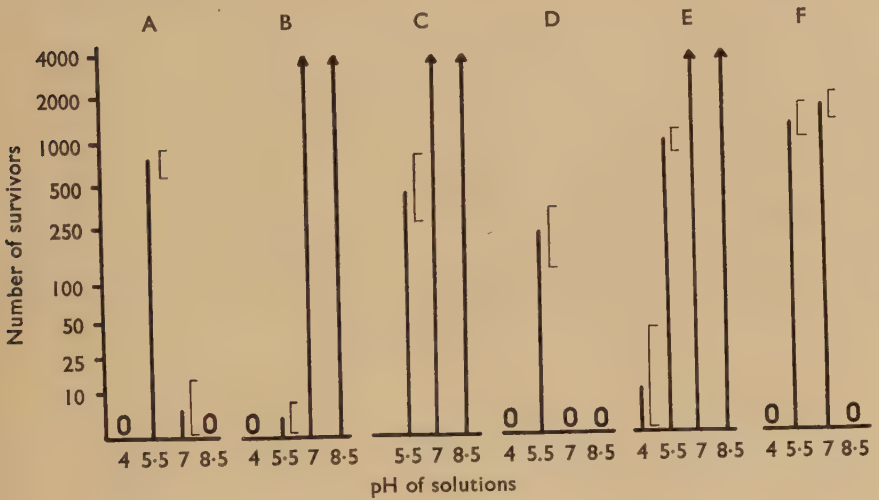


FIG. 1. Membrane filter counts of suspensions of *E. coli* (A, D), *Staph. aureus* (B, E), *Str. faecalis* (C) and *Ps. pyocyanea* (F), exposed to 1 per cent phenol (A, B, C) and 1 per cent (D, E) or 0·5 per cent (F) β-phenylethyl alcohol in isotonic phosphate buffers of four pH values. Each column represents the "mean" calculated on the basis of the logarithms of three parallel counts, brackets indicate lowest and highest counts. An arrow at the top of a column indicates filters too densely grown for a count of single colonies, the limiting count being 2000 to 3000 colonies for the Gram-negative organisms and about 4000 for the Gram-positive ones. For each count, 10 ml. of test-mixture with an initial count of $3 \cdot 10^5$ cells per ml. were filtered, 0 = sterile.

The results with the Gram-negative organisms are contrary to the axiom that phenols are more bactericidal in acid solutions. Especially surprising was that, in general, the efficacy was even more pronounced at pH 8·5 than at pH 4, despite all the phenols tested showing some dissociation at pH 8·5.

One explanation why this characteristic difference between Gram-positive and Gram-negative organisms has not been detected sooner might be that the methods hitherto used e.g., those of Kuroda[2], were not sufficiently sensitive; thus with the more sensitive membrane technique now available these differences are now seen.

As the efficacy of phenols and alcohols was influenced so characteristically by the pH of the solution, we first sought a clue in the different

isoelectric points of Gram-positive and Gram-negative bacteria discussed by Stearn and Stearn[3]. They measured the different uptakes of dye by the cytoplasmic membrane, but to us a difference in the charges of cytoplasmic membranes is difficult to reconcile with the fact that phenols are bactericidal in the molecular form only. It seems that no direct binding of the molecules to acceptor sites can be held responsible; rather, the permeability barrier may be influenced by the external pH. Thus, owing to the difference in barrier between Gram-positive and Gram-negative cells, the penetration of the disinfectants into these cells may not be the same at pH 5·5. Further studies in this field will be necessary to clarify this point.

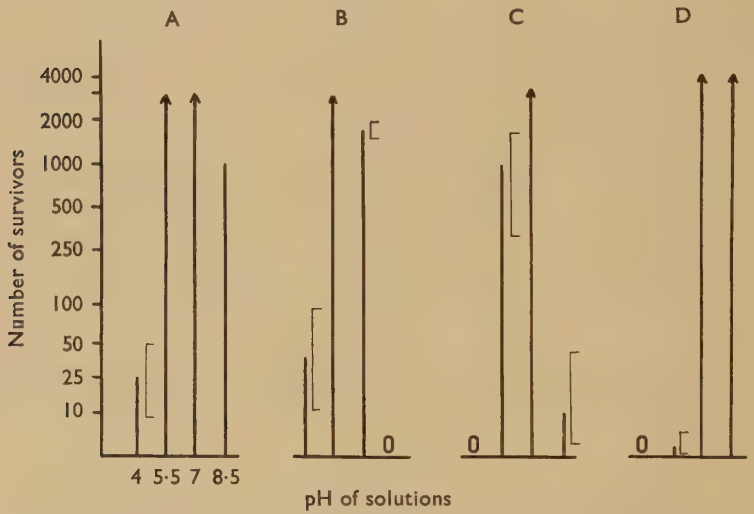


FIG. 2. Membrane filter counts of suspensions of *Serr. marcescens* (A), *Ps. pyocyanea* (B, C, two parallel series), and *Corynebact. diphtheriae* (D), exposed to 0·5 per cent of phenol for 2 hours at 20°. Phosphate buffers, cell density and representation as in Fig. 1.

Influence of the dissociation of phenols. It is well known that th dissociation of phenols increases with increasing pH value, and that this affects their antibacterial properties. This is illustrated in Figure 3 which indicates the bactericidal activities of *p*- and *o*-chlorophenol, propyl-*p*-hydroxybenzoate and *p*-nitrophenol at different pH values against *E. coli*. It shows the rise in dissociation to be proportional to the fall in bactericidal activity. Phenolate ions are therefore not bactericidal, which is in agreement with the statements of other authors. From the Figure, it is possible to estimate the quantitative aspect of the survivor peak at pH 5·5. *o*-Chlorophenol and propyl-*p*-hydroxybenzoate are 3 per cent dissociated at pH 7, which is sufficient to obtain about as many survivors as at pH 5·5. Small differences in concentration seem therefore to be sufficient for a shift in the survivor peak from 5·5 to 7 (or vice versa) with *E. coli*. *p*-Chlorophenol, on the other hand, is

almost 20 per cent dissociated at pH 8·5, with about 20 times as many survivors as at pH 5·5.

The part played by the partition coefficient in the arrangement of the compounds according to their activity. In a preliminary report[4] it was pointed out that our results with phenols and aromatic alcohols are in general agreement with Ferguson's principle[5,6]. This rule, however, stipulating that activity is inversely related to water solubility was not followed by the two chlorinated alcohols, namely, 4-chlorobenzyl alcohol and 4-chloro-β-phenylethyl alcohol. For instance, a 0·021M aqueous solution of 4-chlorobenzyl alcohol (water solubility = 0·021M) exhibited

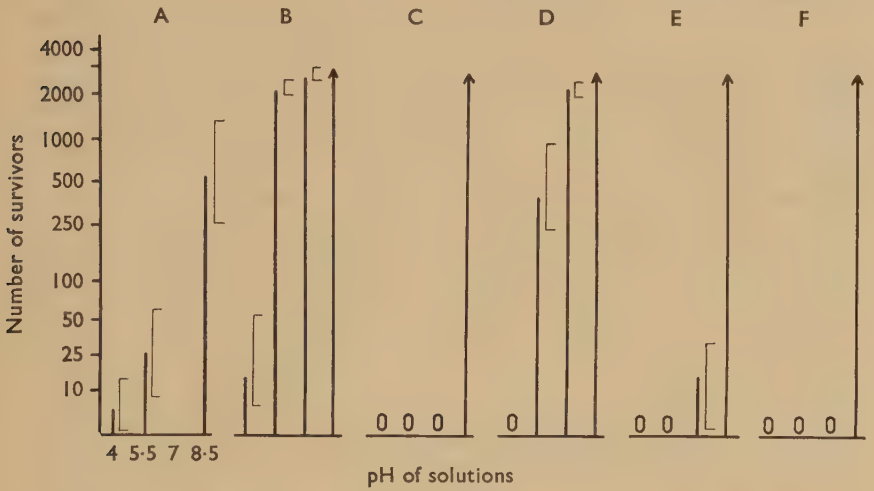


FIG. 3. Membrane filter counts of suspensions of *E. coli* exposed to phenols of varying degrees of dissociation; dissociation rises from left to right. A, *p*-chlorophenol 0·1 per cent for 6 hours at 20°; B, *o*-chlorophenol 0·25 per cent for 30 minutes at 20°, and for 2 hours at 20° in C; D, propyl *p*-hydroxybenzoate 0·03 per cent for 24 hours at 20°; E, *p*-nitrophenol 1 per cent for 30 minutes at 20°; F, *p*-nitrophenol 0·5 per cent for 24 hours at 20°. For buffers, cell density and representation: cf. Fig. 1.

the same bactericidal activity as 0·013M solution of 4-chloro-β-phenylethyl alcohol (water solubility = 0·0294M). However, when these solutions of equal activity were partitioned in hexane: water, 4-chlorobenzyl alcohol achieved a concentration of 0·0106M and 4-chloro-β-phenylethyl alcohol a concentration of 0·0091M in the hexane, the concentrations in the lipid phase thus being similar. Richardson and Reid[7] obtained similar results with α-ω-di-*p*-hydroxyphenylalkanes, as did Shukis and Tallman[8] with aliphatic mercurials. Crisp and Barr[9] confirmed Ferguson's rule giving data on the activities of alcohols and phenols required to immobilise barnacle nauplius larvae. They too stressed that the efficacy of phenols and alcohols is governed by the concentration reached in the biophase. This phase was felt to be a bulk lipid rather polar in character and not an interface between lipid and water.

Our experiments have convinced us that it is possible to use a simple approach to test phenolic and alcoholic disinfectants. Starting from the hypothesis that different concentrations of phenols and aromatic alcohols elicit an identical bactericidal activity when they reach the same molar concentration in a lipid phase after partitioning, reference to physical constants of the compounds in question will allow a comparison of the activities of different compounds to be made. The ratio molar concentration in water : molar concentration in lipid phase after partitioning, provisionally termed "activity index", is a constant for a given compound, provided the same lipid phase is used and that the phenols are undissociated. Once the "activity indices" have been determined experimentally, different compounds can be compared by calculating the concentrations in water which are required to obtain a similar concentration in the lipid phase. The calculated concentrations will probably not be strictly equal in their bactericidal activity since other factors are involved, nevertheless, this approach might be useful for a preliminary comparison.

It has long been known that upon ascending a homologous series, one observes, as a rule, the antibacterial activity to increase and then to decrease again. This fact, already explained in a simple manner by Ferguson[5], may be interpreted from our point of view as follows. On the one hand, the activity of the compounds augments with increasing solubility in lipids. On the other hand, the disinfectant has to be dissolved in water in order to reach the hydrophilic outer layers of the cells. In a homologous series, therefore a certain point will be reached where a further, significant increase in lipid solubility cannot be attained whilst at the same time the solubility in water is lowered, and this means that the activity is necessarily reduced. As Ferguson pointed out, the position of the fall in action depends on the resistance of the organisms; it occurs earlier with *Staph. aureus* than it does with *Salmonella typhi*, for example. One may predict that phenols and aromatic alcohols capable of reaching a high concentration in the cell lipids yet remaining soluble to some extent in water will possess superior killing power. Chlorocresol and 4-chloro-β-phenylethyl alcohol are examples of such compounds.

Our results are in favour of the existence of a lipid barrier at the cell surface. This barrier is very probably represented by the cytoplasmic membrane[10].

Bactericidal Action of Organic Mercury Compounds

Figure 4 shows the activities of phenylmercuric borate and thiomersal at four pH values. It is seen that whilst their activities differ greatly, their effects on the Gram-positive and Gram-negative organisms are identical. It is generally agreed that the mechanism of action of mercurials is an interaction with available sulphhydryl compounds since compounds such as thioglycollate can compete with the mercuric ion. The sulphhydryl group has a pKa of 9·1 to 10·8 at 25° in peptides of known structure[11]. According to Cohn and Edsall[11], the pI (isoelectric point) of glutathione is 2·83 and that of cysteine 5·02 to 5·07; because the more alkaline the

solution of these compounds, the greater their negative charges. The difference between the action of phenylmercuric borate (cationic) and thiomersal (anionic) is easily explained by the fact that in more alkaline solutions a greater number of positively charged phenylmercuric ions can be bound by the sulphhydryl groups; for the negatively charged thiomersal, the opposite is true. The interaction must, of course, occur at a site of the cell still influenced by the external pH. The action of mercurials is further elucidated by results like those of Shukis and Tallman[8], who showed that in a series of aliphatic mercurials, the antibacterial action was better when the compounds had a partition coefficient in favour of

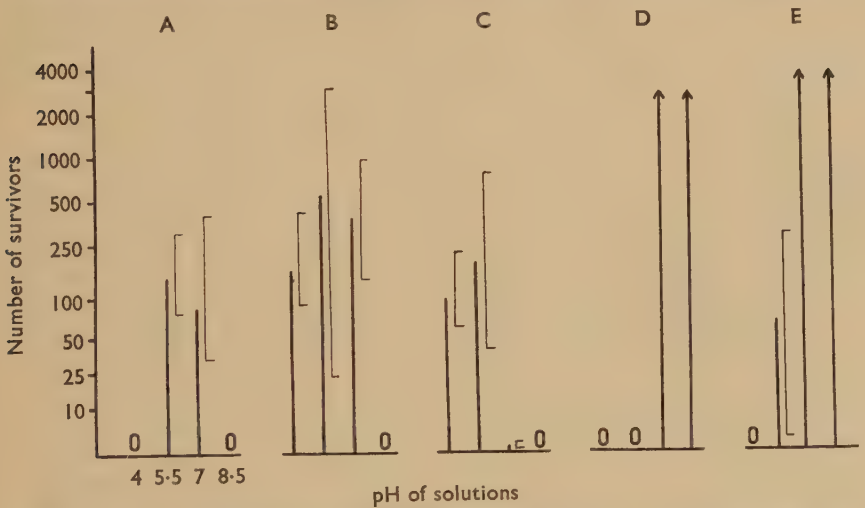


FIG. 4. Membrane filter counts of suspensions of *E. coli* (A, D), *Ps. pyocyanea* (C), and *Staph. aureus* (B, E), exposed to phenylmercuric borate 1:10,000 (A, B, C) and thiomersal 1:10,000 (D, E). Exposure times: 30 minutes at 20° in C, 2 hours at 20° in A and B, 24 hours at 20° in D and E. The phenylmercuric borate solutions were in M/15 phosphate buffers only, the thiomersal solutions were made with the usual isotonic phosphate solutions with potassium chloride. For cell density and representation: cf. Fig. 1.

the lipid phase. We found phenylmercuric borate with its higher solubility in lipids to be much more active than thiomersal which is poorly lipid-soluble.

The action of mercurials seems to proceed in two stages. In the first, the compound is adsorbed on to the cytoplasmic membrane, the adsorption being successful only when the charges of the interacting groups are opposite. The sulphhydryl groups attacked seem to be the same both in Gram-positive and Gram-negative species. The first stage is essentially bacteriostatic and is reversible by thioglycollate. In the second stage, penetration through the lipid barrier, situated most probably in the cytoplasmic membrane, takes place. For this penetration, the water:lipid partition coefficient of the compound is the deciding factor. Once the antiseptic has penetrated, the action is no longer reversible and is therefore bactericidal.

Bactericidal Action of Quaternary Ammonium Compounds

A comparison of the two compounds tested (Figs. 5 and 6) showed that cetyl pyridinium chloride was more active than domiphen bromide against *Staph. aureus*, 1 : 100,000 of the former compound being more effective in 2 hours than the same concentration of domiphen bromide in 24 hours. This difference was never noted with *E. coli*. While a more pronounced

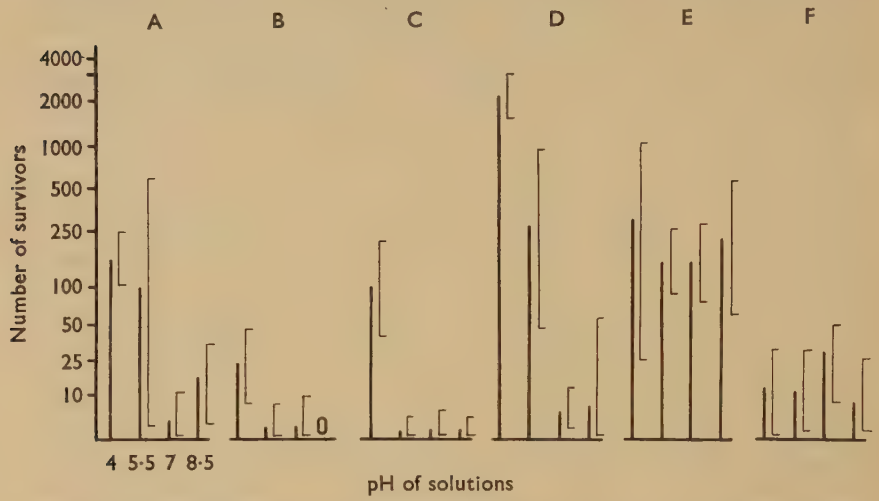


FIG. 5. Membrane filter counts of suspensions of *E. coli* (A, C, E) and *Staph aureus* (B, D, F) exposed to domiphen bromide 1 : 20,000 (A, B), 1 : 50,000 (C, D), and 1 : 100,000 (E, F), Exposure times: 30 minutes at 20° (B), 2 hours at 20° (A, C, D), and 24 hours at 20° (E, F). Experiments B and C were conducted with suspensions of *E. coli* of lesser resistance than those used in A. In experiments B and D, four parallel filtrations were made. For buffers, cell density and representation: cf. Fig. 1.

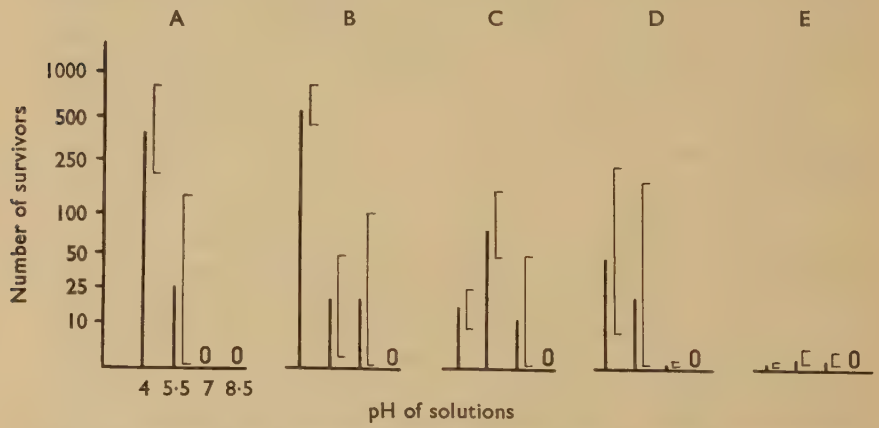
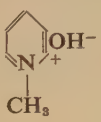
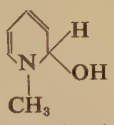


FIG. 6. Membrane filter counts of suspensions of *E. coli* (A, B) and *Staph. aureus* (C, D, E) exposed to cetyl pyridinium chloride 1 : 10,000 for 30 minutes at 20° in A, 1 : 20,000 for 2 hours at 20° in B, 1 : 100,000 for 2 hours at 20° in C, D and E (three parallel series). Buffers, cell density and representation same as in Fig. 1.

bactericidal action of the quaternaries in alkaline solutions is to be expected because of the positive charge of the active molecules, cetyl pyridinium chloride was reported by Quisno and Foter[12] to be equally active between pH 2 to 10. This is partly confirmed by our results (Fig. 6, C-E, in comparison with Fig. 5, D). In spite of its being a weak base (pKb 8·8), pyridine forms quaternary salts which act as strong electrolytes. The quaternary hydroxides, however, exhibit properties[13] that might possibly be able to furnish some explanation for the comparatively milder bactericidal activity at alkaline pH values. Thus, *N*-methylpyridinium hydroxide "a" responds to oxidation with potassium ferricyanide as though it had structure "b".



a



b (Pseudo base)

In alkaline solutions, more of the pseudo base is likely to be formed; this non-ionic compound probably being less bactericidal.

Most workers testing quaternaries have obtained erratic results (see Davies[14]). The explanations generally offered are irregular distribution or agglutination of the cells in the test medium. Combined agglutination and killing have also been advanced to explain the very rapid killing rate in the first few minutes[15,16], followed by a slowing down of the killing process. Davies[14] as well as Du Bois[17] ascribed the persistence of a few survivors to the possibility that cells within agglutinates could no longer be reached by the disinfectant.

To study this further we examined cultures of *E. coli* and *Staph. aureus* in solutions of pH 4 and 7 by phase contrast to see whether agglutination of cells occurred under our experimental conditions. The cell density had to be raised to 15×10^6/ml. for these examinations. *E. coli* was agglutinated by both compounds at 1 : 5000, but not at 1 : 10,000, whereas *Staph. aureus* was agglutinated by dilutions as high as 1 : 200,000. Although agglutination was demonstrated by this method, no differences in survivor counts were found when the cultures were shaken with beads in a lecithin-Tween solution. Our experiments showed the quaternaries to have a very potent bactericidal action which is probably due to the low density of the washed cells employed and to the absence of foreign matter which might act as surface-active adsorbent. The killing of most cells in the first few minutes of exposure is no doubt ascribable to the surface-activity of the quaternaries which are absorbed rapidly by the cytoplasmic membrane, causing disorder and changes in surface charges in this essential part of the cell[18,19]. An important factor seems to be a profound change in permeability. Chaplin[20] described strains of *Serr. marcescens* which were resistant to quaternaries, and he observed that these strains contained more lipids in the cell surface. Similar results were obtained by Dyar[21] with *Staph. aureus*. The persistence of a few survivors might be attributed to differences in the lipid content of the cell surfaces.

Comparison of the Resistance of the Organisms Tested

Our results confirm that *Staph. aureus* is generally more resistant to phenols and aromatic alcohols than *E. coli*. The few experiments with *Str. faecalis* indicate that it is even more resistant than *Staph. aureus*. *Ps. pyocyanea* was more susceptible than *E. coli* to both the phenols and aromatic alcohols as well as to the mercurials and quaternaries tested. However, *Ps. pyocyanea* is still generally regarded as an organism showing marked resistance to disinfectants. Our findings on the efficacy of the quaternaries agree with those of Lawrence[22] and with Ostrolenk and Brewer[23]. Klein and others[24] found the quaternaries to be less active although they used a different method. Berry[25], who tested various organisms against phenoxetol only, suggested that this compound is particularly effective against *Ps. pyocyanea*. However, results of our comparative experiments in which we tested benzyl alcohol, β-phenylethyl alcohol and phenoxetol against *Ps. pyocyanea* show that β-phenylethyl alcohol is the most effective compound. Hence we could not confirm Berry's claim and suggest that the good effect of phenoxetol may be because *Ps. pyocyanea* offers minimal resistance to phenols and aromatic alcohols. After testing a number of typical, freshly isolated strains of the main test organisms we remain convinced that a general high resistance of *Ps. pyocyanea* to disinfectants does not exist although the occurrence of certain highly resistant strains cannot be excluded.

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Curriculum vitae

Am 25. Juni 1930 wurde ich, HANS KASPAR HESS als Sohn des Ernst Hess, Masch.-Ing., von Wald (Zürich) und der Hedwig, geb. Meyerhans, in Basel geboren.

Meine Schulbildung erhielt ich in Basel, zuerst in der Primarschule und dann während acht Jahren im Realgymnasium, wo ich im Frühjahr 1949 die Maturitätsprüfung bestand. Anschliessend begann ich an der Universität Basel das Pharmaziestudium. Nach dem Abschluss des Fachstudiums und bestandenem Staatsexamen im Herbst 1955 begann ich unter der Leitung von Herrn Dr. P. Speiser und Herrn Prof. Dr. K. Meyer die vorliegende Dissertation, die ich im Sommer 1958 abschloss.

Während meines Studiums besuchte ich die Vorlesungen und Praktika folgender Herren Professoren und Dozenten: H. Baur, H. Erlenmeyer, M. Geiger-Huber, P. Huber, E. Iselin, H. Lehmann, K. Leupin, K. Meyer, T. Reichstein, E. Rothlin, J. Tomcsik, I. Tschudi-Steiner und W. Vischer.